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Publication date	2017-05-17
Original Citation	Csepregi, K., Coffey, A., Cunningham, N., Prinsen, E., Hideg, É. and Jansen, M. A. K. (2017) 'Developmental age and UV-B exposure co-determine antioxidant capacity and flavonol accumulation in Arabidopsis leaves', Environmental and Experimental Botany, 140, pp. 19-25. doi:10.1016/j.envexpbot.2017.05.009
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1016/j.envexpbot.2017.05.009
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Download date	2023-05-07 18:28:04
Item downloaded from	http://hdl.handle.net/10468/4025

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PII: S0098-8472(17)30113-2
DOI: http://dx.doi.org/doi:10.1016/j.envexpbot.2017.05.009
Reference: EEB 3232

To appear in: *Environmental and Experimental Botany*

Received date: 31-3-2017
Revised date: 12-5-2017
Accepted date: 15-5-2017

Please cite this article as: Csepregi, K., Coffey, A., Cunningham, N., Prinsen, E., Hideg, É., Jansen, M.A.K.,Developmental age and UV-B exposure co-determine antioxidant capacity and flavonol accumulation in Arabidopsis leaves, *Environmental and Experimental Botany* (2017), http://dx.doi.org/10.1016/j.envexpbot.2017.05.009

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- In Arabidopsis rosettes, developmental age of leaves modulates UV-B responses.
- Low UV-B positively affects UV-absorbing pigments, flavonols and total antioxidants.
- Developmental age affected photochemistry, and especially energy dissipation
- Developmental age associated variation in UV-absorbing pigments and antioxidant activity can exceed the response induced by low UV

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Developmental age and UV-B exposure co-determine antioxidant capacity and flavonol accumulation in Arabidopsis leaves

Running title

Developmental age and UV-B acclimation in Arabidopsis

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Abstract

Developmental age is an important determinant of plant stress responses. In this study the importance of "within-individual-heterogeneity" of developmental age for plant UV-B responses was quantified. *Arabidopsis thaliana* rosettes were raised under indoor conditions, and the responses of leaves at different developmental stages were compared following exposure to supplemental UV-B radiation. Exposure to a low dose of UV-B had positive effects on concentrations of UV-absorbing pigments, quercetins and kaempferols as well as total antioxidant activity measured. Unlike UV-B, developmental age had a substantial effect on photochemistry, and especially energy dissipation. Younger leaves display relatively strong regulated dissipation, while older leaves show more non-regulated, non-photochemical energy dissipation. Developmental age also impacted on concentrations of UV-absorbing compounds, and antioxidant activity. In fact, developmental variation matched, or even exceeded the UV-induced response for these two parameters. Thus, pooling of rosette leaves is not necessarily a good strategy to visualise plant UV-responses. Rather, to fully understand plant UV-responses in a developmental context it is important to advance reporter technologies for physiological studies, including spin-trap technology to visualise *in planta* ROS and ROS-defences, and fluorescence excitation screening technology and chromogenic assays for *in planta* visualisation of specific UV-absorbing pigments.

Key words

Antioxidant, Arabidopsis, development, flavonol, photochemistry, Ultraviolet-B

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Introduction

Ultraviolet-B (UV-B) radiation is an important regulator of plant growth and development (Jansen and Bornman 2012; Hideg *et al*., 2013; Jenkins, 2014; vanHaelewyn *et al*., 2016). Amongst others, UV-B controls accumulation of a broad range of plant secondary metabolites with UV-screening and/or antioxidant activities (Jansen *et al*., 2008, Zhang and Björn 2009). Many studies have reported the UV-induced accumulation of flavonols and related phenolics (Searles *et al*., 2001; Bieza and Lois 2001; Rozema *et al*., 2002; Jansen *et al*., 2008). These compounds occur in cell walls, in the vacuoles of mesophyll cells (Kytridis and Manetas 2006, Agati *et al*., 2009), in chloroplasts (Agati *et al*., 2007) and in non-secretory, and glandular trichomes (Tattini *et al*., 2007). Additionally, there is good evidence that UV-mediates the accumulation of terpenoids, alkaloids, glucosinolates, polyamines and tocopherols (Jansen *et al*., 2008, Zhang and Björn 2009, Schreiner *et al*., 2012). It is likely that many, if not all, of these UV-induced metabolites contribute to Reactive Oxygen Species (ROS) scavenging capacity and/or UV screening, and therefore to UV protection. Thus, plant responses to low doses of UV-B are typically acclimative (i.e. driven by eustress), resulting in increased UV-protection. In contrast, high doses of UV-B are associated with distress, i.e. metabolic disruption (Hideg *et al*., 2013), and such distress can be quantified as accumulation of DNA-dimers (Britt 1996), inactivation of photosynthetic activity (Jordan *et al*., 2016), and/or as macroscopic damage such as chlorosis. Whether UV causes eustress or distress does not simply depend on the UV dose, but rather on the balance between damaging reactions, repair and acclimation responses (Jansen *et al*., 1998). An important determinant of repair and acclimation responses, and hence net plant damage, is plant and leaf developmental age.

Plant developmental age is an important determinant of stress susceptibility. Amselem *et al*., (1993) showed that resistance to the ROS generator paraquat peaked at week 10, and then gradually decreased with developmental stage in

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paraquat-resistant *Conyza bonariensis*. It has been shown that 3-week-old *Arabidopsis thaliana* rosettes respond differently to stressors such as low temperature, and wounding than 6-week old rosettes. Young plants showed higher *Pal1* transcript accumulation than older plants in response to low temperatures, while older plants showed stronger induction of peroxidase activity (Abarca *et al*., 2001). Similarly, analysis of antioxidants in Arabidopsis rosettes ranging in age from 19 through to 75 day's old showed marked differences in ascorbate, glutathione, NAD and NADP levels with developmental age (Queval and Noctor, 2007). Thus, the literature emphasises the importance of plant developmental age for plant stress responses.

Few studies have considered "within-individual heterogeneity" in developmental age, when studying plant stress. Individual plants represent complex temporal and spatial mosaics of organ developmental age and stress susceptibility (Coleman, 1986). The association between organ age and stress susceptibility appears to be due to a range of physiological, biochemical and structural characteristics that distinguish organs of different developmental ages. Thus, exposure of the entire plant to a stressor triggers a heterogeneous mixture of responses and it has been argued that for many leaves maximal stress susceptibility occurs at the time of the sink-source transition (Coleman, 1986). Although, the importance of leaf developmental age has been recognised as a source of "within-individual heterogeneity" in stress responses (cf, Coleman, 1986), there is no simple standardised approach to quantify leaf developmental age. Common approaches to quantifying leaf development include numbering successive leaves, measuring percentage of full leaf expansion, and/or measuring time from leaf initiation (Coleman, 1986). However, within-individual heterogeneity of stress responses is not just related to leaf age. Plants comprise complex, 3-dimensional structures giving rise to different microclimates. In the case of UV-B exposure, younger leaves positioned near the top of the plant are exposed to ambient light conditions, while the exposure of older leaves depends on light penetration in the 3-D structure of the plant. As the diffuse fraction of UV-B irradiance is larger than that of visible wavelengths, the UV: PAR ratio may increase within the plant

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canopy (Yang *et al*., 1993; Brown *et al.,* 1994). The resulting interaction between positional effects and developmental age has not been well analysed. Some studies have analysed the composition and antioxidant activity of tea (*Camellia sinensis*) leaves of differing developmental age. Younger leaves were found to have higher total phenolic content and antioxidant activity (Chan *et al*., 2007), and higher theanine and caffeine content, but lower catechin concentrations (Song *et al*., 2012). Similarly, younger leaves of blackberry, raspberry and strawberry were also found to have higher total phenolic content and antioxidant activity compared to older leaves (Wang and Lin, 2000). However, while these data are important from an agronomical perspective, they fail to separate developmental effects from positional light-exposure effects. For experimental purposes, positional UV-exposure effects can be avoided by selecting plants with a 2-D structure. For example, a study of leaf developmental stage and UV-responses in grapevine (*Vitis vinifera*, cv. Chardonnay) used single shoots which were led along horizontal rods. Upward-facing leaves occurred in a sequence from oldest to youngest leaves, and all leaves were equally exposed to ambient light (Majer and Hideg, 2012).

Rosettes are another attractive system to study within-individual heterogeneity, in the absence of major positional, micro-climatic effects. *Arabidopsis thaliana* rosettes, especially those at the early stages of development with just a few true leaves (see Boyes *et al*., 2001 for nomenclature), are quite flat and display little self-shading. Several studies have shown that Arabidopsis leaf developmental age will affect accumulation of secondary metabolites. For example, glucosinolate concentrations are higher in younger Arabidopsis leaves than in older leaves (Brown *et al*., 2003). However, little is known about the importance of leaf developmental age for the accumulation of flavonols, total antioxidant capacity and UV-protection, and the relationships between these three parameters. Here, it is hypothesised that leaf development is a determinant of plant UV-responses. If this is hypothesis is confirmed, the question should be asked whether (commonly practised) harvesting of entire rosettes for UV-acclimation studies is scientifically justified. The aim of the current study was to probe the

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interdependence between UV-B acclimation and leaf developmental processes in *Arabidopsis thaliana,* by measuring photosynthetic efficiency, total antioxidant capacity, UV-absorbing pigments and specific flavonols.

Materials and Methods

Plant growth and UV-exposure

Seeds of *Arabidopsis thaliana* Columbia-0 were vernalized at 4^0C before sowing into flats containing sieved John Innes No.2 compost. The flats were covered in cling film and placed in a temperature controlled growth room with a 16 hour light and 8 hour dark photoperiod. Once the seeds had germinated the cling film was removed. Seedlings were raised under 70 µmol m^{-2} s^{-1} PAR. At the two cotyledon stage the seedlings were transplanted into individual pots containing John Innes No. 2 compost. The seedlings were allowed to reach the 1.04 growth stage (Boyes *et al*., 2001) before the experimental treatment was initiated. Leaves were numbered in order of developmental age, with the first-formed (i.e. oldest) leaves counted as numbers 1 and 2.

UV-exposure experiments were conducted in a self-contained light box, fitted with fluorescent tubes emitting Photosynthetic Active Radiation (PAR) (36W Philips Master TLD Reflex Tube), UV-A (Philips Fluorescent Blacklight Blue 36W, 1200mm) and UV-B (Philips TL12). Temperature within the box was 22^0C and the relative humidity was 55%. The PAR intensity was 60-80 µmol/m^{-2}s, and this was supplemented by 1.6W/m^2 UV-A. A dimmable ballast (Sylvania-Biosystems, Wageningen, The Netherlands) was used to regulate the intensity of the TL12 tubes without changing the UV-B spectrum (verified with Ocean Optics Spectroradiometer (USB2000+RAD) (Ocean Optics, Dunedin, FL, USA). The output of the UV-B tubes was set to generate 0.6W/m^2. Plants were exposed to UV-B radiation for two hours each day at noon, for a total of 7 days. This translates to a biological effective dose of 0.6648kJ m^{-2} day^{-1} (Flint and Caldwell, 2003). The UV-C component that is generated by the TL12 tubes was blocked using a filter of cellulose acetate (95µm thickness; Kunststoff-Folien-Vertrieb GmbH, Hamburg, Germany). Control plants (no UV-B) were grown under UV-B blocking filter (125µm thickness, Polyester film, Tocana Ltd.,

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Elizabeth's Cross, Ballymount Cross Ind. Est., Ballymount, Dublin 24). Both filters were placed 5cm above the plants on opaque frames. Both filters were changed after 20 hours of UV-B exposure. The photoperiod in the light box was the same as the growth room, a 16 hour light and 8 hour dark sequence. The plants were acclimated to the light box for a minimum of 24 hours before switching on the UV-B tubes.

Chlorophyll a *fluorometry*

Chlorophyll *a* fluorescence (Fv/Fm; Y(II), Y(NPQ), Y(NO) and NPQ) parameters were determined using an Imaging PAM (Waltz, Effeltrich, Germany). Whole rosettes were dark adapted for a minimum of 20 minutes before the maximal quantum yield of Photosystem (PS) II F_v/F_m was determined. Following this, leaves were exposed to non-modulated blue actinic light (186 µmol m^{-2} s^{-1}) and light acclimated minimum and maximum fluorescence yields were obtained using a saturating pulse. Light acclimated PS II yield Y(II), regulated non-photochemical quenching Y(NPQ) and non-regulated non-photochemical quenching Y(NO) were calculated from fluorescence parameters according to Klughammer and Schreiber (2008). NPQ was calculated as Y(NPQ)/Y(NO). Photosynthetic activities were determined for leaves 1 to 7 from 5 independent replicate rosettes.

Total soluble phenolics

Total soluble phenolics were extracted from leaves numbers 1 to 7 using acidified methanol (1%HCL, 20%H_2O, 79%CH_3OH). Whole leaves were placed in micro-tubes containing acidified methanol and incubated in the dark at 4^0 for 4 days. The supernatant was drawn off using a pipette and placed in quartz glass curvette. Absorbance was recorded at 330nm on a spectroradiometer (Shimadzu UV-160A) and normalized against fresh weight. A total of 5 independent replicates were used for each leaf.

Analytic quantification quercetin and kaempferol glycosides

Glycosylated quercetin and kaempferol compounds were quantified in leaves 4, 5 and 6. Each independent replicate comprised leaves from at least 5 plants,

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which were pooled to provide enough biomass for UPLC analysis, for each treatment. Arabidopsis leaves were frozen using liquid nitrogen and ground in a Magnalyser (5x 15 sec, 6500 rpm, Roche diagnostics, Vilvoorde, Belgium). To extract flavonols, leaves were homogenized in acidified methanol (0.125% FA, 62.5 % MeOH, 5 µl per mg fresh weight) and sonicated in an ultrasonic bath for 30min followed by filtration (True Nylon Syringe filter, 0.2 µm, Grace Davison Discovery Science, Deerfield, IL, USA).

Flavonol compounds were analysed using an ACQUITY UPLC chromatography system combined with and ACQUITY TQD mass spectrometer. The solvents used were water, 0.1% formic acid (C) and acetonitrile, 0.1% formic acid (D). TQD analysis was performed in ESI(+)-MRM mode. Concentrations were measured using a mass spectrometer, and calculated following calibration against the reference compound kaempferol-3-rhamnosidoglucoside (10^{-5}M final concentration, Carl Roth GmbH, Karlrühe, Germany). For the purpose of the experiments, the main UV-responsive quercetin and kaempferol glycosides were identified. These were kaempferol-3-*O*-glucoside-7-*O*-rhamnoside; kaempferol-3-*O*-glucosyl-glucoside-7-*O*-rhamnoside; kaempferol-3-*O*-rhamnoside-7-*O*-rhamnoside; kaempferol-3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside; quercetin-3-*O*-glucoside-7-*O*-rhamnoside; quercetin-3-*O*-rhamnoside-7-*O*-rhamnoside; quercetin-3-*O*-glucosyl-glucoside-7-*O*-rhamnoside; and quercetin-3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside. Their concentrations were combined to give total kaempferol-glycosides and quercetin-glycosides, respectively. There were 5 independent replicates for flavonol measurements, and each replicate was comprised of the leaves of a further 5 plants.

Trolox equivalent antioxidant capacity (TEAC)

2,2□-Azino-bis (3-ethylbenzothiazoline-6-sulfonic cation radical (ABTS•+) reduction was measured based on the method of Re *et al*., (1999) as described earlier (Csepregi *et al*., 2016). $ABTS^{\bullet+}$ was prepared by mixing 0.1 mM ABTS, 0.0125 mM horse radish peroxidase and 1 mM H_2O_2 in a 50 mM phosphate buffer (pH 6.0). After 15 min, 10 µL diluted leaf extract or test compound was

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added to 190 µL $ABTS^{\bullet+}$ solution and conversion of the cation radical into colourless ABTS was followed as decrease in absorption at 651 nm recorded with a Multiscan FC plate reader (Thermo Fischer Scientific, Shanghai, China). Myricetin-3-*O*-glucoside was used to prepare a calibration curve and TEAC of leaf extracts were given in reference to that of Myricetin-3-*O*-glucoside. There were 4 independent replicates of each sample, and each replicate contained pooled biomass from 10-15 plants.

Ferric reducing antioxidant power (FRAP)

FRAP is based on detecting the capacity of samples to reduce ferric ions, which is measured as an absorbance change of the ferrous 2,4,6-tripyridin-2-yl-1,3,5-triazine (TPTZ) complex. The assay was carried out according to a modification (Szőllősi and Szőllősi-Varga, 2002) of the original medicinal biochemical assay (Benzie *et al*., 1996). The FRAP reagent was prepared by mixing 25 mL of acetate buffer (300 mM, pH 3.6), 2.5 mL TPTZ solution (10 mM TPTZ in 40 mM HCl) and 2.5 mL of $FeCl_3$ (20 mM in water solution). For each sample, 10 µL diluted leaf extract or test compound was added to 190 µL freshly mixed FRAP reagent. Samples were incubated in microplate wells at room temperature for 30 min before measuring the OD at 620 nm using a Multiscan FC plate reader (Thermo Fischer Scientific, Shanghai, China). FRAP values of were expressed as µmol Myricetin-3-*O*-glucoside equivalents per mg leaf dry weight. There were 4 independent replicates of each sample, and each replicate contained pooled biomass from 10-15 plants.

Data analysis

Effects of leaf age and UV-B radiation were the two factors and the above metabolic, antioxidant or photosynthetic parameters were variables in statistical analyses. Effects of leaf age and UV-B were analysed using two factor ANOVA. The null hypothesis was that neither leaf age nor UV-B were effective. When a significant age or UV-B effect was identified based on a $p < 0.05$ result, Tukey's post-hoc tests were carried out to explore connections between individual factors and variables. Pair wise relationships of parameters were tested further using simple linear regression. Linear fits were characterized by

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the regression coefficient R^2 and p values of t-tests using the null hypothesis that the slope of the fitted regression line was zero. This hypothesis was rejected for data sets with $p < 0.05$ and these were concluded to be linearly correlated. Calculations were carried out using the PAST statistical software (Hammer *et al*., 2001).

Results

Arabidopsis thaliana rosettes were exposed to a low dose of UV-B radiation for 7 days after which rosettes were dissected and individual leaves assayed for photosynthetic competence, total UV-absorbing content, kaempferol and quercetin content, and antioxidant and radical scavenging capacity.

Leaf photochemistry and non-photochemical quenching

The maximum quantum yield of PSII (Fv/Fm) was not significantly affected by UV-B treatment or developmental age (Table 1). Rather, measured values (0.77 on average) indicate a good activity of PSII throughout all samples. The quantum yield of PSII under steady state conditions (Y(II)), and the quantum yield of regulated non-photochemical energy loss in PS II (Y(NPQ)) are similarly not affected by the used dose of supplemental UV-B radiation. Although there appears to be a slight increase in Y(II) and Y(NPQ) in younger leaves in UV-B exposed rosettes, this is not significant. In contrast, the non-photochemical quenching (NPQ) shows a developmental aspect, with significantly higher values in younger leaves. The quantum yield of non-regulated non-photochemical energy loss in PS II (Y(NO)) does show significantly lower values in younger leaves, although no UV-B effects are discernible.

UV-absorbing pigments and flavonols

The content of soluble UV-absorbing pigments in methanolic extracts was determined and normalised as a function of leaf fresh weight. The leaves of rosettes that had been exposed to supplemental UV-B contained consistently higher levels of UV-absorbing pigments (Fig. 1). Compared to non UV-B exposed controls, levels of UV-absorbance increased by some 67% in UV-

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exposed leaf 1, but just 16% in case of leaf 7. A significant effect of leaf age on UV-absorbing pigment content was also noted. On average, non UV-exposed leaf 7 contains 67% more UV-absorbing pigments than non UV-exposed leaf 1. Interactions between UV and developmental age were not significant.

UPLC/MS was used to separate and quantify levels of quercetin-, and kaempferol-glycosides in *Arabidopsis thaliana* leaves (Table 2). Analysis was limited to leaves 4, 5 and 6, for which enough biomass could be generated. Concentrations of both quercetin and kaempferol strongly increased in response to UV-B exposure. For example, quercetin levels in leaf 5 were nearly 10-fold higher in a UV-B exposed leaf, relative to the non UV-B exposed control. Increases in kaempferol were more modest, with a 3.7-fold increase in kaempferol content in UV-B exposed leaf 5 compared to the non-UV exposed control. There is no significant developmental effect on leaf flavonol content, although it is noted that lowest concentrations of the glycosylated flavonols occur in leaf 4, and highest in leaf 6. Across all data (leaves 4, 5 and 6; + or – UV-B) quercetin concentrations are positively correlated with kaempferol concentrations (Fig. 2A). Similarly, both quercetin and kaempferol concentrations are positively correlated with the UV-absorbance of methanolic extracts (Fig. 2B, 2C).

Total antioxidant activity

Trolox equivalent antioxidant capacity (TEAC) was determined by quantifying the reduction of the ABTS-cation in *Arabidopsis thaliana* leaves 3, 4, and 5. To generate enough biomass, leaves 1 and 2, and leaves 6 and 7 were combined. On average, the leaves that had been exposed to supplemental UV-B radiation displayed significantly higher TEAC-values (Fig. 3A). The decrease in TEAC-values with increasing (i.e. younger) leaf number was not significant. Measurements of ferric reducing antioxidant power (FRAP) display a different developmental pattern, with significantly increasing FRAP-values with increasing leaf number. UV-B exposure further significantly enhanced FRAP-values, especially in the newest leaves (Fig. 3B).

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Correlations between quercetin and kaempferol concentrations and Ferric Reducing Antioxidant Power (FRAP) were investigated across different leaves and inclusive of both UV-exposed and control samples (Fig. 4A, 4B). A positive relation was identified, whereby the highest flavonol content coincides with the highest FRAP activity. Whilst both kaempferol and quercetin content are positively correlated with FRAP values, only quercetin correlates positively with TEAC values. No correlation was found between FRAP and TEAC values.

Discussion

Effects of UV-B radiation on Arabidopsis thaliana leaves

UV-exposure had a significant, positive effect on the total content of UV-absorbing pigments. UV-B induced increases in total phenolic content have been reported in large numbers of studies (e.g. Searles *et al*., 2001; Bieza and Lois 2001; Rozema *et al*., 2002; Jansen *et al*., 2008). We noted a positive correlation between increased absorbance of methanolic extracts and kaempferol and quercetin-glycoside contents (Fig 2B and 2C). Thus, increases in UV-absorbance of methanolic extracts are most likely due to increases in the concentration of flavonols and related pigments. This does, however, not exclude contribution by hydroxycinnamic acids and their esters to total UV-absorbance of leaves.

Quercetin concentrations did increase up to 10-fold in UV-exposed leaves, while increases in kaempferol concentration were more modest (3-4 fold). Such differential regulation of different flavonol compounds has been observed before (cf. Hideg *et al*., 2013) and is thought to reflect the higher ROS-scavenging capacity of quercetins with their additional hydroxyl group on ring-B, relative to kaempferol (Csepregi *et al*., 2016). Increases in UV-induced total antioxidant capacity (FRAP) are consistent with the rise in flavonols, with their strong antioxidant activity (Hernandez *et al*., 2009), and a positive correlation can be observed between the total antioxidant capacity of leaf extracts assessed as FRAP and quercetin or kaempferol concentrations (Fig. 4A and 4B). Remarkably, no UV-mediated increases in TEAC were observed in this study, notwithstanding the significant correlation between TEAC and quercetin

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content. Thus, significant increases in total UV-absorbing pigments, and in quercetin and kaempferol glycosydes did not result in an increase in total antioxidant activity measured using the TEAC assay. These data are in agreement with a study by Csepregi *et al.*, (2016) who showed that different antioxidant assays can yield different results when used to compare phenolic-rich samples. Csepregi *et al.*, (2016) have argued that differences in the reactivities of quercetin and kaempferol derivatives with the chromophores of the two TAC assays underlie such diversity in measured responses (Csepregi *et al.*, 2016). This, together with the clear developmental effect on FRAP, which is absent for TEAC, indicate that TEAC and FRAP assays measure different aspects of plant antioxidant defences.

The upregulation of the total content of UV-absorbing pigments and the total antioxidant activity (FRAP) in UV-exposed leaves is indicative of UV-acclimation. Consistently, in this study we show that plants exposed for 7 days to supplemental UV-B do not show significant negative effects on the maximal yield of photosystem II (Fv/Fm), on the steady state yield (Y(II)), and on non-photochemical quenching. Many older studies have reported negative effects of UV-B radiation on photosynthesis, including O_2 evolution, chlorophyll *a* fluorescence, CO_2 fixation, stability of the D1 and D2 protein core of photosystem II and stomatal function (Jordan *et al.*, 2016), however where realistic UV-doses and/or exposure conditions are used, most studies indicate minor UV-effects on the photosynthetic machinery. Using realistic exposure conditions, Wargent *et al.*, (2015) even demonstrated a positive effect of UV-radiation on photosynthesis by measuring net carbon fixation. However, in this study the relatively low UV-B doses did neither have a positive, nor a negative effect on photochemistry.

Leaf developmental age as a determinant of leaf photosynthesis, pigment composition and total antioxidant activity

In this study leaves of different developmental age were compared. Arabidopsis leaves are well known for developmental age related changes which are visible as heteroblasty, i.e. the age dependent changes in the

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morphology of leaves including shape and trichome distribution (Tsukaya *et al*., 2000). In this study it was found that developmental age had no effects on either Fv/Fm or Y(II). However, leaf developmental age was a significant determinant of both Y(NO) and NPQ, with lower Y(NO) and higher NPQ values in younger leaves. Thus, younger leaves display relatively strong regulated dissipation through, for example, the xanthophyll cycle. In contrast, older leaves display more non-regulated, non-photochemical energy dissipation. Previously, Majer and Hideg (2012) showed negative effects of UV radiation on the photochemical yield of older leaves, while younger leaves were more protected. A comparison of "inner" (i.e. younger) versus "outer" (i.e. older) leaves of 6 week old Arabidopsis plants also showed that older leaves displayed UV-B mediated impairment of photosystem II, unlike younger leaves (Jordan *et al*., 1998). Although no developmental UV-effects on photosynthetic activities were observed in this study, our data support the concept that young and old leaves have different photoprotection strategies.

Clear effects of leaf developmental age were also seen in the study of total soluble phenolic content, where an increase in UV-absorbance of methanolic extracts was observed in younger leaves (Fig. 1). Previously, Majer and Hideg (2012) reported that young grapevine leaves display the strongest increases in UV-absorbing pigments, anthocyanins, total phenolics and total antioxidant capacity in response to supplemental UV. A comparison of "inner" (i.e. younger) versus "outer" (i.e. older) leaves of 6 week old Arabidopsis plants also showed stronger upregulation of UV-absorbing pigments in younger leaves (Jordan *et al*., 1998). These data are in agreement with a generalisation proposed by Harper (1989), who argued that young, expanding leaves depend on phenolics for defence purposes, while older leaves may defend themselves through a tough cuticle and/or high content of dry matter, both of which are incompatible with rapid growth. However, the link between leaf developmental age and leaf phenolic content is not that straightforward. In *Ilex paraguariensis* total phenolic content increases with leaf ageing (Blum-Silva *et al*., 2015), an observation which contradicts the data observed in this study. In *Lantana camara* levels of total phenolics were stable across a range of leaves of

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different developmental ages (Bhakta and Ganjewala, 2009) but concentrations of specific flavonols displayed a bell shaped curve, with lowest levels in young and mature leaves. Thus, the composition of the total pool of UV-absorbing pigments changes with developmental age in *Lantana camara*. Reifenrath and Müller (2007) who found higher concentrations of flavonols in young, compared to old leaves in *Sinapsis alba* and *Nasturtium officinal*. Similarly, Bergquist *et al*., (2005) observed higher levels of flavonols in young, compared to more mature plants of baby spinach (*Spinacia oleracea*). In this study, a non-significant increase in flavonol content with developmental age was observed. Some of the variation in published data on total phenolics and/or flavonols can be due to variations in experimental approaches, including extraction and quantification procedures (see Julkunen-Tiitto *et al*., 2015). Indeed, esterified and other forms of covalently cell wall bound phenolic compounds are notoriously difficult to quantify, and it can't be excluded that the proportion of such compounds varies with developmental age. Most studies, including this one, are therefore limited to the extractable phenolic compounds. Yet, some of the contradictory information on phenolic concentrations is likely to be accurate and visualise species-specific responses. A comparison of young and old leaves of eight different species showed that in four species older leaves contained more total phenolics, in three species younger leaves contained more phenolics, and in one species older and younger leaves contained similar amounts of phenolics (Achakzai *et al*., 2009). The species in which older leaves contain more phenolics include the deciduous shrub *Berberis vulgaris*, the deciduous tree *Melia azedarach*, and the evergreen shrubs *Nerium oleander* and *Rhododendron sp*. The species in which younger leaves contain more phenolics include the evergreen trees *Olea europea* and *Tamarix aphylla,* and the deciduous, invasive tree *Prosopis glandulosa*. As all species were analysed in the same laboratory, using the same techniques, it is likely that the developmental age dependency of phenol accumulation is species specific.

The data presented in this paper show developmental age related changes in total antioxidant capacity, and UV-absorbance of methanolic extracts

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concentrations. Thus, sampling a single "representative" leaf on a plant does not necessarily capture the full scope of "within-individual-heterogeneity". In the context of plant UV-studies, we note that the developmental variation in the UV-absorbance of methanolic extracts and in total antioxidant activity matches, or is even greater than the measured UV-induced response. Thus, pooling of rosette leaves is not necessarily a good strategy as specific leaves may skew averages (consider for example effects of different kaempferol concentrations in Table 2). Superficially, it appears that the best strategy to comprehensively visualise UV-responses is to analyse all individual leaves within a rosette. However, such an approach does not consider the importance of "within-leaf-heterogeneity" for plant UV-responses, including differences between "older distal" leaf zones and "younger proximal" zones, and between "more UV exposed adaxial epidermal tissue" and "less exposed mesophyll tissue". In the field of gene-expression studies, analysis of the tissue (or even cell) specificity of response has long been facilitated by reporter-constructs. This study emphasises that to fully understand plant UV-responses in a developmental context, it is important to further develop reporter technologies for physiological studies, including high sensitivity imaging techniques to visualise ROS and ROS-defences *in planta*, as well as fluorescence excitation screening technology and chromogenic assays for *in planta* visualisation of specific UV-absorbing pigments.

Acknowledgements

AC and MAKJ acknowledge financial support from Science Foundation Ireland (11/RFP.1/EOB/3303 to MAKJ). KCs and ÉH acknowledge financial support from the Hungarian National Research, Development and Innovation Office through the Hungarian Scientific Grant Agency (OTKA K112309 to ÉH). EP acknowledges financial support from the Fund of Scientific Research Flanders (G000515N).

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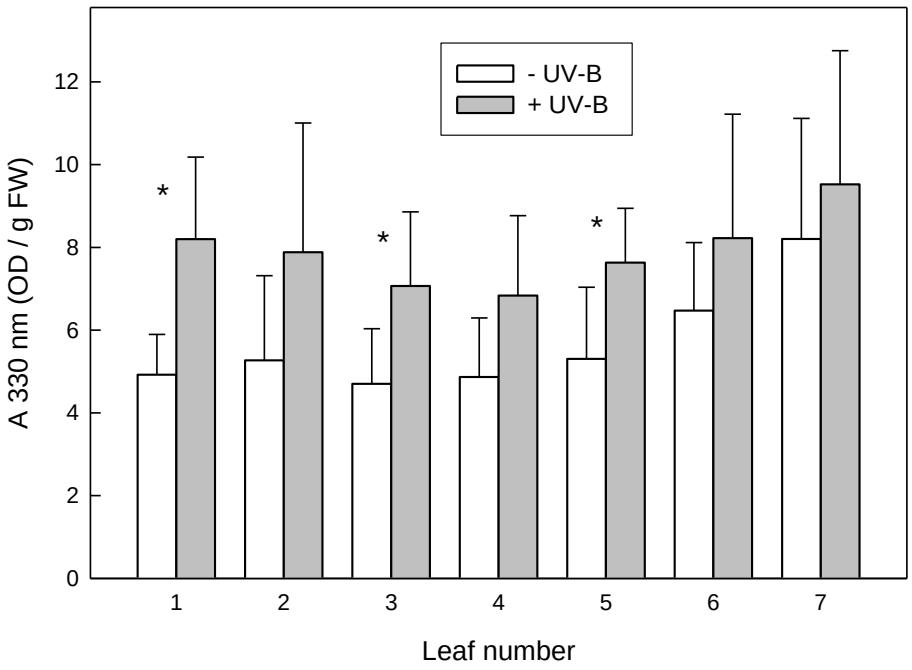

Figure 1: Total UV-absorbing pigments (A330 nm/g fresh weight) as a function of leaf number. Leaves were numbered in order of their formation, with leaves one and two being the oldest leaves in the rosette. UV-absorbing pigments were extracted using acidified methanol. Plants had either been grown under PAR plus UV-A (indicated as –UV-B), or under PAR plus UV-A plus UV-B (indicated as +UV-B). Data are means ± standard deviations. N=5. Two-way ANOVA revealed UV-B ($p<0.01$) and leaf age ($p<0.05$) as significant factors in determining the content of total UV-absorbing pigments. Asterisks mark significant ($p<0.05$) different means.

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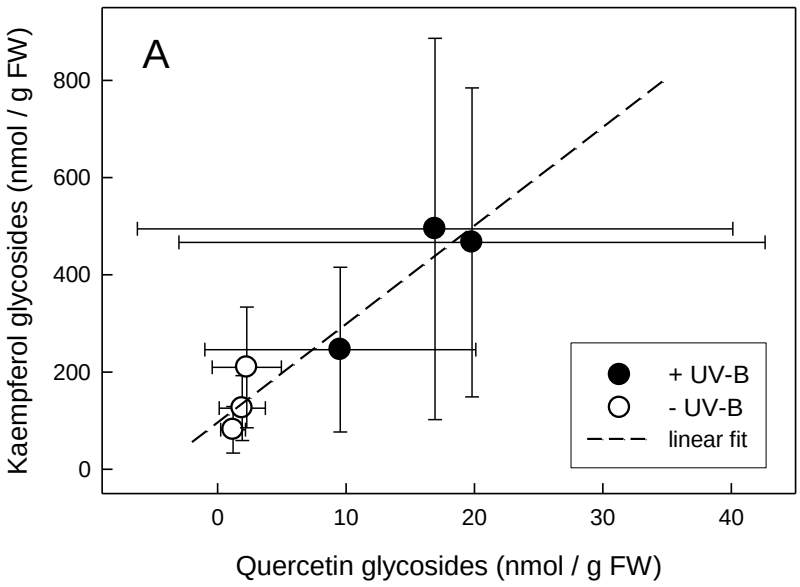

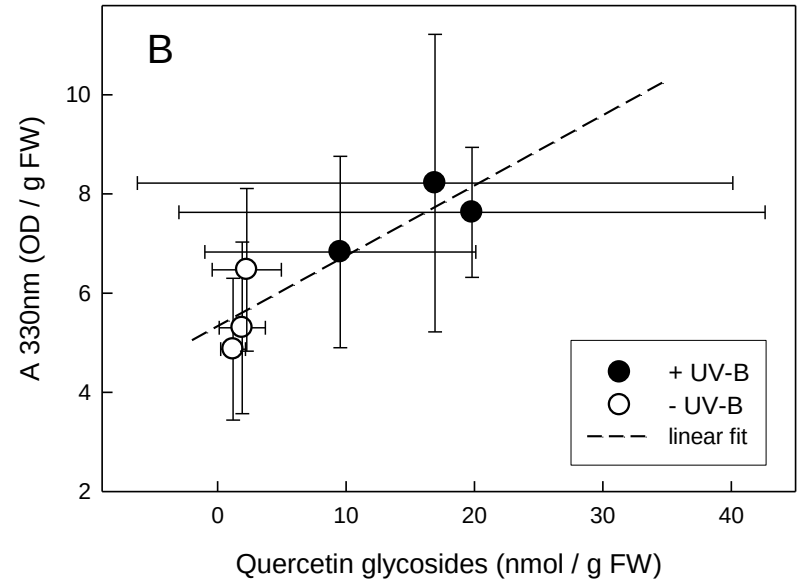

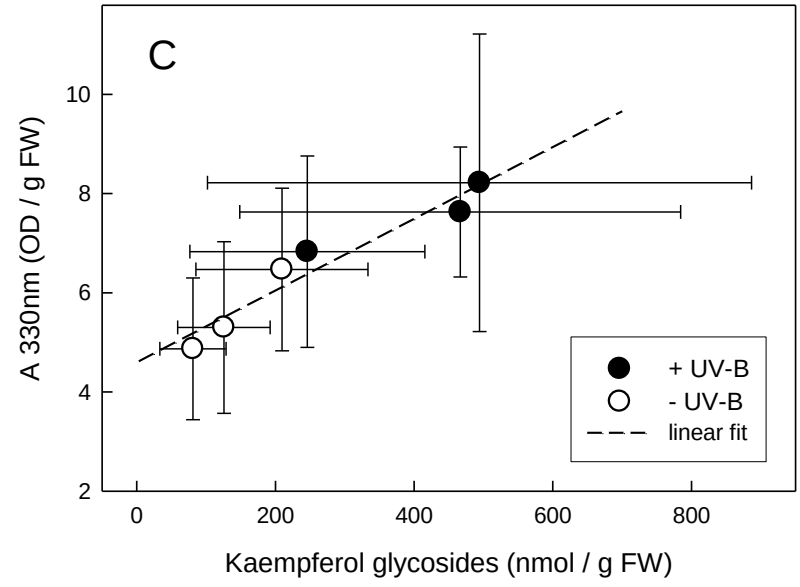

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Figure 2: Relationships between kaempferol-glycosides, quercetin glycosides and total UV-absorbing pigments (A330 nm/g fresh weight) in Arabidopsis leaves 4, 5 and 6. Plants had either been grown under PAR plus UV-A (indicated as –UV-B), or under PAR plus UV-A plus UV-B (indicated as +UV-B). Panel A, quercetin versus kaempferol content; B, quercetin versus A330nm; C kaempferol versus A330nm. Data are means ± standard deviations (n=5). Dashed lines show linear fits of either whole data sets (30 data pairs for panel A) or averages (6 data pairs for panels B and C). Fig.2A: R^2= 0.912, $p < 10^{-5}$; Fig.2B: R^2= 0.501, $p = 1.22\ 10^{-5}$; Fig.2C: R^2= 0.538, $p < 10^{-5}$.

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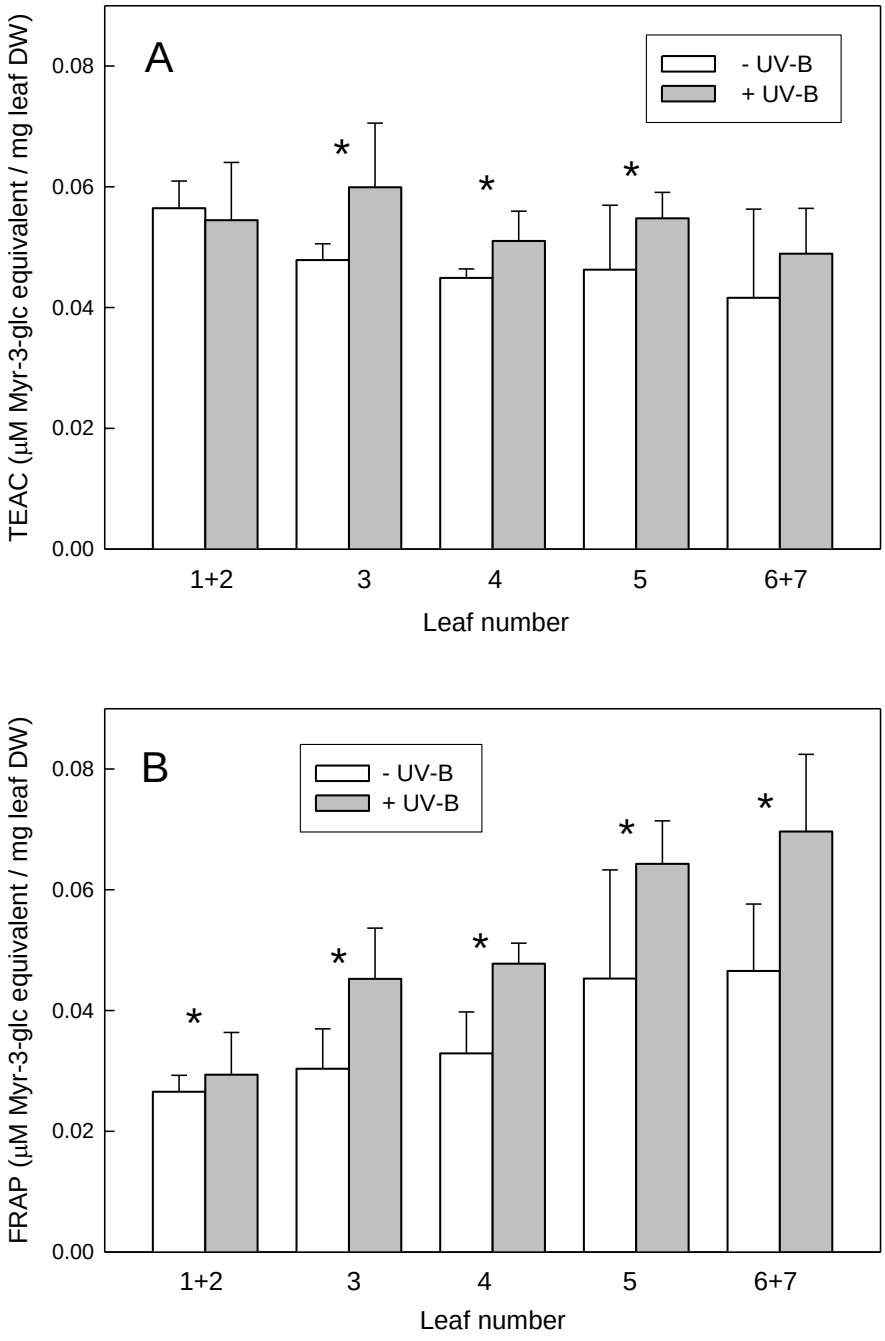

Figure 3: Total antioxidant capacity as a function of leaf number. Leaves were numbered in order of their formation, with leaves one and two being the oldest leaves in the rosette. Total antioxidant capacity was measured using the TEAC assay (panel A) or the FRAP assay (panel B). Plants had either been grown under PAR plus UV-A (indicated as –UV-B), or under PAR plus UV-A plus UV-B (indicated as +UV-B). Data are means ± standard deviations. N=4. Asterisks mark significant ($p<0.05$) different means. Two-way ANOVA revealed UV-B ($p<0.05$) as a significant factor for TEAC, and both UV-B ($p<0.01$) and leaf age ($p<0.01$) as significant factors for FRAP.

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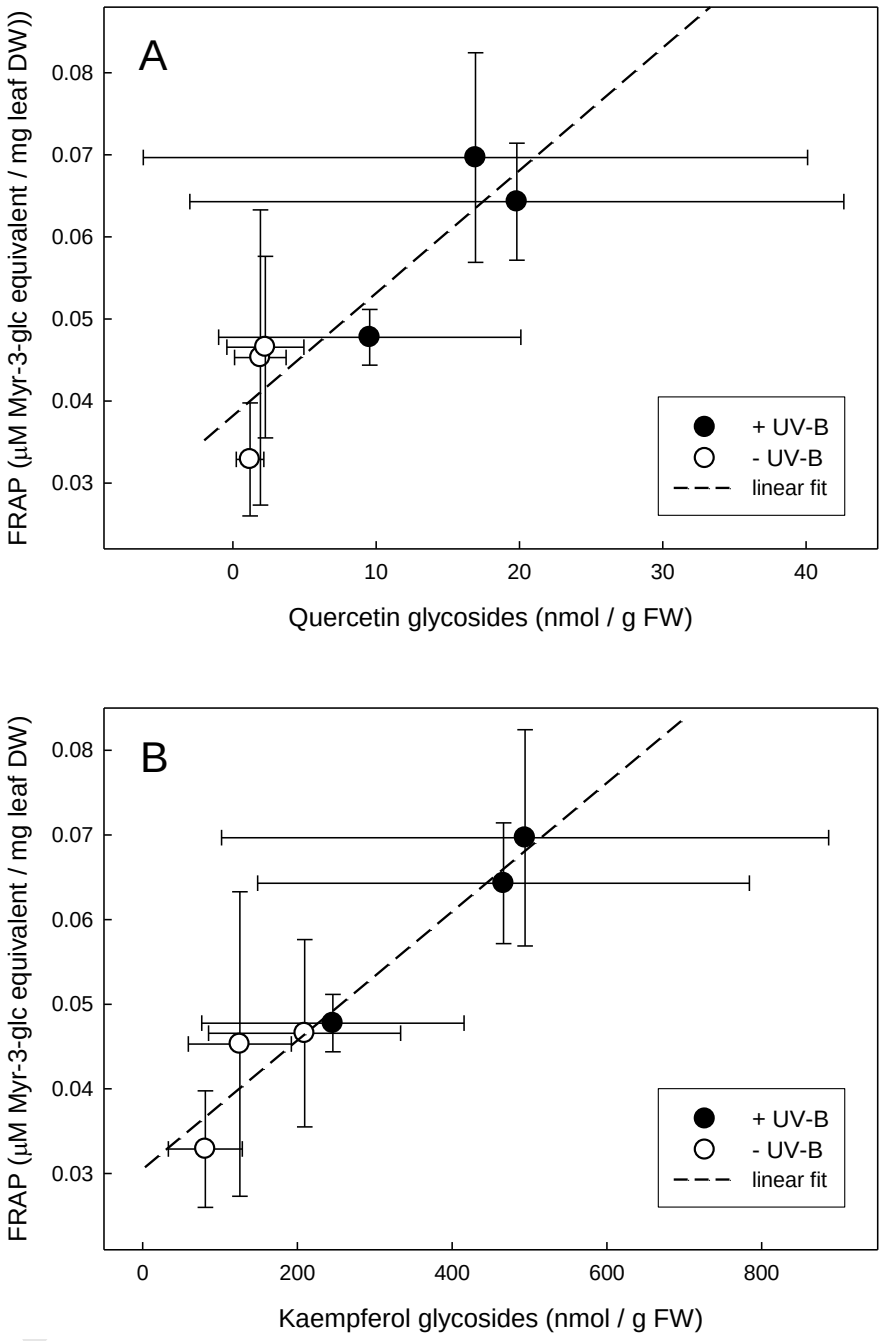

Figure 4: Relationships between kaempferol-glycosides, quercetin glycosides and total antioxidant activity (FRAP) in Arabidopsis leaves 4, 5 and 6. Plants had either been grown under PAR plus UV-A (indicated as –UV-B), or under PAR plus UV-A plus UV-B (indicated as +UV-B). Panel A, quercetin versus FRAP; B, kaempferol versus FRAP. Data are means ± standard deviations. N=5 for quercetin and kaempferol measurements, and n= 4 for FRAP. Dashed lines show linear fits using averages (6 data pairs). Fig.4A: R^2= 0.813, p= 0.013; Fig.4B: R^2= 0.946, p = 0.001.

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Table 1: Photosynthetic parameters of individual leaves of an *Arabidopsis thaliana* rosette, measured using chlorophyll a fluorometry. Plants had either been grown under PAR plus UV-A (indicated as –UV-B), or under PAR plus UV-A plus UV-B (indicated as +UV-B). Shown are averages of 5 independent replicates, with standard deviations. *significant effect of leaf age ($p<0.01$).

Leaf number	Fv/Fm		Y(II)		NPQ*		Y(NPQ)		Y(NO)*	
	-UV-B	+UV-B	-UV-B	+UV-B	-UV-B	+UV-B	-UV-B	+UV-B	-UV-B	+UV-B
1	0.78±0.03	0.76±0.03	0.17±0.06	0.18±0.06	0.73±0.13	0.75±0.18	0.35±0.05	0.35±0.04	0.48±0.04	0.47±0.07
2	0.78±0.02	0.77±0.02	0.19±0.07	0.17±0.07	0.70±0.11	0.66±0.13	0.33±0.05	0.33±0.05	0.48±0.04	0.50±0.05
3	0.78±0.02	0.77±0.02	0.20±0.04	0.22±0.09	0.82±0.16	1.03±0.47	0.36±0.05	0.38±0.12	0.44±0.04	0.40±0.09
4	0.77±0.01	0.77±0.03	0.21±0.04	0.22±0.07	0.94±0.09	0.96±0.26	0.38±0.03	0.38±0.08	0.41±0.03	0.40±0.06
5	0.76±0.05	0.77±0.02	0.22±0.07	0.23±0.07	1.03±0.16	0.86±0.14	0.39±0.04	0.36±0.05	0.39±0.06	0.42±0.05
6	0.76±0.03	0.77±0.04	0.22±0.09	0.24±0.11	1.30±0.34	1.15±0.51	0.44±0.09	0.40±0.13	0.34±0.03	0.36±0.06
7	0.76±0.05	0.77±0.05	0.20±0.16	0.25±0.13	1.45±0.41	1.30±0.47	0.48±0.15	0.42±0.14	0.33±0.01	0.33±0.02

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Table 2: Quercetin and kaempferol content measured using LC-MS. Plants had either been grown under PAR plus UV-A (indicated as –UV-B), or under PAR plus UV-A plus UV-B (indicated as +UV-B). Data are means ± standard deviations. N = 5. *Two-way ANOVA of the whole data set identified UV-B but not leaf age as a significant factor for both total quercetin-glycoside (F=7.08, p=0.013) and kaempferol-glycoside (F=10.22, p=0.004) content.

Leaf number	**Quercetin nmol/g FW**		**Kaempferol nmol/g FW**	
	-UV-B	+UV-B*	-UV-B	+UV-B*
4	1.2±1.0	9.6±10.6	81.1±47.8	246.0±169.4
5	1.9±1.8	19.8±22.8	125.7±66.7	466.5±317.8
6	2.3±2.7	16.9±23.2	209.4±124.1	494.4±392.3

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